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Cloning of zebrafish activin type IIB receptor (ActRIIB) cDNA and mRNA expression of ActRIIB in embryos and adult tissues

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Abstract

A full-length cDNA encoding for activin type IIB receptor (ActRIIB) was cloned from zebrafish embryos. It encodes a protein with 509 amino acids consisting of a signal peptide, an extracellular ligand binding domain, a single transmembrane region, and an intracellular kinase domain with predicted serine/threonine specificity. The extracellular domain shows 74–91% sequence identity to human, bovine, mouse, rat, chicken, *Xenopus* and goldfish activin type IIB receptors, while the transmembrane region and the kinase domain show 67–78% and 82–88% identity to these known activin IIB receptors, respectively. In adult zebrafish, ActRIIB mRNA was detected by RT-PCR in the gonads, as well as in non-reproductive tissues, including the brain, heart and muscle. In situ hybridization on ovarian sections further localized ActRIIB mRNA to cytoplasm of oocytes at different stages of development. Using whole-mount in situ hybridization, ActRIIB mRNA was found to be expressed at all stages of embryogenesis examined, including the sphere, shield, tail bud, and 6-7 somite. These results provide the first evidence that ActRIIB mRNA is widely distributed in fish embryonic and adult tissues. Cloning of zebrafish ActRIIB demonstrates that this receptor is highly conserved during vertebrate evolution and provides a basis for further studies on the role of activin in reproduction and development in lower vertebrates.

Keywords: Activin IIB receptor; Cloning; mRNA expression; Ovary; Embryos; Zebrafish

1. Introduction

Activin is a growth and differentiation factor belonging to the transforming growth factor β (TGFβ) family (reviewed by Mathews, 1994). Activin exists in at least three isoforms, activin A, activin B and activin AB, which result from dimerization of two independent gene products: βA and/or βB subunits (Mathews, 1994). Although activin was originally isolated from porcine follicular fluid based on its ability to stimulate pituitary follicle-stimulating hormone secretion, it is now clear that it functions as an autocrine/paracrine regulator in a variety of tissues (Mathews, 1994). There is increasing evidence which indicates that activin is involved in many physiological processes, particularly in reproduction and development. Activin has been shown to regulate: (1) gonadal hormone production and gametogenesis (Hutchinson et al., 1987; Itoh et al., 1990; Mather et al., 1990; Mauduit et al., 1991; Li et al., 1995; Peng et al., 1996); (2) placental hormone production (Qu and Thomas, 1995; Petraglia et al., 1996); (3) neuronal cell survival (Schubert et al., 1990); (4) proliferation of a variety of cell types (Kojima and Ogata, 1989; McCarthy and Bicknell, 1993); (5) erythropoiesis (Eto et al., 1987); and (6) induction of mesoderm formation during early embryonic develop-

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ment (van den Eijnden-Van Raaij et al., 1990; Sokol and Melton, 1991).

The biological effects of activin are mediated through specific activin receptors. Molecular cloning and biochemical studies have demonstrated that a functional activin receptor complex consists of both type I and type II receptors (Mathews, 1994; Massagué, 1996). Activin first binds to the type II receptor, forming an active complex that is able to bind with the type I receptor. The type II receptor kinase *trans*-phosphorylates the type I receptor, which subsequently activates downstream cellular signals (Massagué, 1996). A novel family of proteins, consisting of Mad from *Drosophila* (Sekelsky et al., 1995), Sma from *C. elegans* (Savage et al., 1996), and the vertebrate homologs, Smads (also known as Madr or hMad) (Baker and Harland, 1996; Chen et al., 1996; Hu et al., 1998), have been identified as intracellular mediators of activin and other members of the TGFβ family. Molecular cloning and characterization of activin receptors have revealed that there are multiple subtypes of both type I and type II receptors. In mammals, two type I (ActRIA and ActRIB) (Tsuchida et al., 1993; ten Dijke et al., 1994; Xu et al., 1994), and two type II (ActRIIA and ActRIIB) (Mathews and Vale, 1991; Attisano et al., 1992) activin receptors have been identified.

Although most studies on activin have been conducted using mammals, several lines of evidence have suggested that activin is also involved in the reproduction and development of teleost fish. Molecular cloning of activin βA and βB subunits from goldfish (Ge et al., 1993b, 1997a) and the βB subunit from zebrafish (Wittbrodt and Rosa, 1994) reveals that the activin structure is highly conserved during vertebrate evolution. In goldfish, activin-A stimulates gonadotropin-II (GTH-II) secretion from the pituitary in vitro (Ge et al., 1992). Our recent studies have demonstrated that activin-A promotes germinal vesicle breakdown of zebrafish oocytes, suggesting that activin-A is involved in the induction of final oocyte maturation (Garg and Peng, unpublished data). Activin has also been shown to induce the expression of several genes which are the early markers for mesoderm induction, such as *axial* (Strähle et al., 1993) and *Sna-1* (Hammerschmidt and Nusslein-Volhard, 1993). Furthermore, overexpression of dominant negative mutants of activin disrupted mesoderm and axis formation in Japanese medaka (Wittbrodt and Rosa, 1994).

Zebrafish is emerging as a model for vertebrate development. It provides the possibility of combining embryology and genetics to address developmental questions. In addition, a short generation time of 3 months also makes zebrafish an ideal model to study the function of genes using transgenic approaches. As the first step to use molecular biology tools to further study the role of activin and its receptors in fish reproduction, we have cloned the ActRIIB from a zebrafish embryonic cDNA library and demonstrated its mRNA expression in embryos, as well as in adult tissues.

2. Materials and methods

2.1. Zebrafish

Adult zebrafish were obtained from a local aquarium and maintained in the laboratory according to Westerfield (1995). Experiments were performed according to the 'Guide to the Care and Use of Experimental Animals' by Canadian Council on Animal Care.

2.2. Screening of a cDNA library

Construction of the cDNA library from embryos at 6–72 h stages has been reported previously (Gong et al., 1997). By partial sequencing of randomly selected cDNA clones from a zebrafish embryonic cDNA library (Gong et al., 1997), a 2.3 kb clone, termed E254, was found to have a high degree of identity with known ActRIIBs. Since this clone represents the 3′-end sequence, PCR was performed to obtain a probe for cDNA library screening. An antisense primer complementary to a region at the 5′-end of the E254 clone (5′ACAGACCTGAATGCTT 3′) and a sense primer which is conserved in all mammalian species (5′ TTCTGCTGCTGTGAAGGAAACT 3′) (Hildén et al., 1994), were used in PCR to amplify ActRIIB cDNA (Fig. 1). A product with the expected size of 1.1 kb was purified and ligated into pT7Blue T-vector (Novagen Inc., Madison, WI). One of the clones, A1, was selected, sequenced and found to be highly similar to

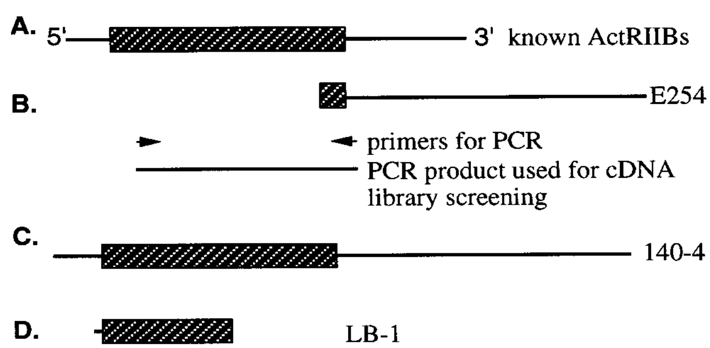

Fig. 1. Cloning strategies and isolated clones. (A) Schematic structure of ActRIIB. Lines represent untranslated regions and the box area is the coding region. 2) Clone E254 was isolated by partial sequencing of randomly selected cDNA clones from a zebrafish embryonic cDNA library. (B) Clone A1 is the PCR product obtained using a primer derived from the conserved region of known ActRIIB and a primer complementary to the 5′-end sequence of the E254. (C) Clone 140-4 was isolated through cDNA library screening using A1 as a probe. (D) Clone LB-1 was obtained during the random isolation of zebrafish maternal cDNAs.

ActRIIB cloned from mammals. The insert from clone A1 was labeled with ^{32}P α-dATP (Amersham, Oakville, ON) using a Random Primers cDNA Labeling System (Life Technologies Inc., Burlington, ON). Hybridization was carried out under high stringency conditions as previously described (Peng et al., 1994). Approx. 2 million phages were screened and a positive clone with a 3.6 kb insert (140-4) was purified and sequenced. Another clone, termed LB-1, was obtained during a random isolation of zebrafish maternal cDNAs from a cDNA library constructed using pre-midblastula transition (< 250-cell) zebrafish embryo mRNA (Bally-Cuif et al., 1998). Upon sequencing, it was found that this clone contains 863 bp corresponding to nt 337–1200 of clone 140-4 (Fig. 2).

2.3. Sequencing analysis

Clones 140-4, E254, and LB-1 were initially sequenced from both strands using T3 and T7 primers. Subsequently, internal primers were made based on the sequence of the clones and further sequencing analyses were carried out. For direct sequencing of PCR products, after electrophoresis, DNA fragments were excised and recovered using Geneclean Kit (Bio101, Vista, CA), and sequenced using PCR primers as well as internal primers. All sequencing was performed using a ABI 373A Sequencer at York University's Core Facility for Molecular Biology. Analysis of the sequencing data was performed using Blast and GCG programs.

2.4. RNA isolation, reverse transcription and PCR

Zebrafish were anesthetized with MS222 (Sigma, St. Louis, MO) and sacrificed by decapitation. Brain, heart, liver, kidney, skeletal muscle, ovary and testis were then removed. Total RNA was isolated from these tissues using the TRIzol reagent (Life Technologies Inc.), according to the manufacture's suggestion. Two micrograms of total RNA were reverse transcribed using the First Strand cDNA Synthesis Kit and oligo-$dT_{12\text{-}18}$ primers (Pharmacia), as previously described (Peng et al., 1994). Two primers, ActRIIB3 and ActRIIB2 (Fig. 2), located in the coding region were used in the PCR to examine the distribution of ActRIIB mRNA in adult zebrafish tissues. Three sets of primers, ActRIIB10 + ActRIIB2, ActRIIB4 + E254R4, and E254F2 + ActRIIB8 (Fig. 2), were used to confirm the coding sequence and the 3′ untranslated region (3′UTR). PCR was carried out in the presence of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 50 μM dNTPs, 1 U Taq DNA polymerase (Life Technologies) or *pfu* DNA polymerase (Stratagene, La Jolla, CA) and 10 pmol primers. Thirty-five cycles (denaturing at 94°C for 20″; annealing at 60°C for 30″ and extension at 72°C for 50″ (for ActRIIB3 + 2) were performed on a DNA Thermal Cycler 2400 (Perkin-Elmer, Norwalk, CT). To confirm the identity of PCR products, DNA fragments separated in agarose gels were transferred to nylon membranes (Amersham) and hybridized with the A1 cDNA probe (for primers ActRIIB3 and ActRIIB2). PCR products from all other primer combinations were subjected to direct sequencing.

2.5. In situ hybridization

Clone LB-1 was subcloned into pBluescript SK +. Both sense and antisense probes were labeled using digoxigenin (DIG)-11-UTP (Boehringer Mannheim). For in situ hybridization of ovarian sections, ovaries were collected from anesthetized females, fixed overnight at 4°C in 4% paraformaldehyde, rinsed in 0.12 M phosphate buffer, dehydrated in ethanol and butanol, and embedded in paraplast plus at 60°C. Sections of 7–10 μm were collected on Superfrost-plus slides and subjected to hybridization as described in Johnston et al. (1997). The oocyte stages were determined according to Selman et al. (1993). For whole-mount in situ hybridization, embryos obtained from natural crosses were staged according to Kimmel et al. (1995). They were then dechorionated and fixed as described above. Hybridization was performed under high stringency conditions according to Thisse et al. (1993) with minor modifications; such as hybridization was performed at 68°C instead of 70°C and incubation with anti-dig antibody was carried out at room temperature for 2 h instead of overnight at 4°C.

3. Results

3.1. Cloning of zebrafish ActRIIB

By partial sequencing of randomly selected cDNA clones from a zebrafish embryonic cDNA library, a 2.3 kb clone (E254) showing high homology with ActRIIBs from other species was obtained. The clone was fully sequenced at both strands. Among the 2348 nucleotides, 245 represent the coding sequence and the rest is the 3′ UTR. To obtain the 5′ end sequence of zebrafish ActRIIB, PCR was performed on a zebrafish embryonic cDNA library using an antisense primer derived from E254 and a sense primer conserved in all mammalian species studied. An expected 1.1 kb PCR product was obtained, cloned and subsequently used as a probe for cDNA library screening. After screening approximately 2 million phages, a positive clone (140-4) with a 3.6 kb cDNA insert was successfully obtained. As shown in Fig. 2, the clone has 3598 nucleotides, which contains an open reading frame of 1530 base pairs encoding for a protein of 509 amino acids. An-

(a)

1 GCACGAGCGG GCGACCAGAA GCTGCACAGG CGGACAGGCT CTGCAAGACC
51 TGCCCTGAGA TTTGGTTGAT TATTTGGGGG GCGTCAGTGT GGATTATCCG
101 CCGTTCCTGA TGGCTTTAAA TCGGGCTCAG GTTTGACTGA GAGCCCCTGG
151 TGTGGACATC AGGCCGACGG TCACTCGGGC TCCAGCGCGC GCGTGTGCGT
201 GTGGTGTGTG AGAGTGTGTG TGTGTTTTTC AGTGAGTGAG TGCGTGTGAG
ActRIIB10
251 TGTGTGATCG GTTTAAATGT GTGCGAGGCA GAGGACGCCG AACAGGAGTA
301 AATCCGGGCT GTTTTTGGTC TGATTAAAGG AAT**ATG**TTCG CTTCTCTGCT
ActRIIB3
351 CACTTTGGCA CTTCTTCTGG CAACTTTCGC TGCAGACCCC AGTCATGGCG
401 AGGTGGAGAC GCGGGAGTGT TTGTACTATA ACGTTAACTG GGAGGTGGAG
451 AAGACGAACC GTAGCGGTGT GGAGCGATGT GAAGGAGAGA AAGACAAACG
501 CTCGCACTGC TACGCCTCCT GGAGGAACAA CTCCGGCTCC ATCCAGCTCG
551 TCAAGAAGGG CTGCTGGCTC GACGACTTCA ACTGCTACGA CAGGCAGGAG
601 TGTGTGGCCA CAGAGGAGAA TCCTCAGGTG TTTTTCTGCT GCTGTGAAGG
651 AAACTTCTGC AATGAGAGAT TCACACACCT GCCGGACATC AGTGGACCAG
701 TGATCTCTCC TCCGCCAGTG TCTCCATCTC TGCTGAATGT GTTGGTTTAC
751 TCTCTGCTGC CGCTCTCCAT GCTCTCCATG GCTGTGCTGT TGGCATTCTG
801 GATGTACCGA CACAGAAAAC CTCCGTACGG ACATGTGGAC GTCAATGAGG
ActRIIB4
851 ATCCAGGCCC ATCTCCTCCA TCTCCTCTGG TGGGTCTGAA GCCTCTGCAG
901 CTGCTGGAGG TTAAAGCTCG CGGACGTTTC GGCTGCGTCT GGAAGGCTCA
951 GATGATCAAT GAATATGTAG CTGTCAAGAT TTTCCCCATT CAGGATAAGC
1001 TGTCGTGGCA GAACGAGCGG GAGATGTTTT CCACTCCGGG AATGAAACAT
1051 GATAACCTGC TGCGCTTCAT CGCTGCTGAG AAACGCGGAT CTAACCTGGA
1101 GATGGAGTTC TGGCTCATCA CTGAATTTCA TGAGCGGGGC TCTCTGACGG
1151 ACTATCTGAA GGGGAACGCA GTGAGCTGGG CTGATCTGTG TGTGATAGCG
1201 GAGAGCATGG CCTGTGGTCT GGCGTATCTG CATGAAGACG TGCCGCGATC
1251 CAAAGGAGAA GGCCCCAAAC CAGCCATCGC ACACAGAGAC TTTAAGAGCA
ActRIIB2
1301 AGAATGTGAT GCTGAAGATG GACCTCACCG CCGTCATTGG GGATTTTGGG
1351 CTGGCGGTGC GGTTTGAGCC GGGGAAACCG CCGGGAGACA CACATGGCCA
1401 GGTGGGCACG AGGAGGTACA TGGCCCCGGA GGTGCTGGAA GGAGCCATAA
1451 ACTTCCAGCG GGACTCCTTT CTGCGGATAG ACATGTACGC CATGGGCCTG
1501 GTGCTGTGGG AGCTGGTGTC ACGCTGCAAA GCTGCTGATG GTCCTGTGGA
1551 CGAGTACATG CTGCCGTTTG AGGAGGAGAT CGGTCAGCAC CCGTCGCTGG
1601 AGGATCTGCA GGATGCTGTG GTCCATAAGA AGCTGCGGCC GGCGTTTAAG
1651 GACTGCTGGC TCAAGCATTC AGGTCTGTGT CAGATGTGCG AGACCATGGA
1701 GGAGTGCTGG GATCATGACG CAGAGGCTCG TCTGTCGGCC GGCTGTGTGC
1751 AGGAGCGCAT CTCTCAGATC CGCCGCGTCA GCAGCTCCAC CTCAGACTGC
1801 CTGTTCTCCA TGGTGACCTC GCTCACCAAC GTGGACCTGC CGCCCAAAGA
1851 GTCCAGCATC **TGA**ACACACT CAGAGAAGAA CAAAACAAAC ACACACACCA
1901 CCCTCAAGCA GCTGCTATTT TACCACGACC CTTTTTTTAA ACTGTCCTCG
1951 TTACTGTTGT TTATTATTAT TATTATTATT ATGATTTTTA TTTTTCTGAT

Fig. 2. Nucleotide sequence of zebrafish ActRIIB. Nucleotides 1-2700 are derived from clone 140-4 and 2701-3963 are from clone E254. Underlined regions are sequences found only in E254 but not 140-4. Several primers used in PCR studies are indicated by overlines. The sequence presented here has been submitted to Genbank under the accession number of AF069500.

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(b) 2001  CGGATCAGCA ACTTTACCAG CACACTTACT CTTCTCTACT GTATTTTTAT
    2051  CATCGGAGCA AACGCGACGA AGCGTGCATT CAGGTGCCGA CGAATGAATG
    2101  CTGACGCTGC AGGTACCTCA AGGTTTATCT GGTGTTGTGT TTTTTCCTCT
                          E254R4
    2151  TCTTCTGGAT GGCTGAGCAG TGCTGCAGAC CCGCGGGGAT CATCATGACT
    2201  GTTACACAAA GCTAGCTCCT CCGGGTTTAT TTTCTCTCTC TCCGACAGTG
    2251  AAACGTGTCT TTCGGAGCTA ACGGGTGTTC AGGAACACCA CACCACAGCC
    2301  GGCAGGTTTT GGATGATCTT GTGGCGGTGT CTCTCTCACG GCTGGCTTGC
    2351  GTGTGCAGAT TTCGGAGCAT TTAGTTCGGT ACAGGAATCT TTCTCAGGGA
    2401  TCCAGAGTCG AGGGGTCCAT GACTGCCTTT TTTTCCGCAT CAGTATAGGA
                                     E254F2
    2451  CAGAGATCGG TTTATAATGC CAATTACCTA CCAGGGCTGG GACCATAAAT
    2501  CATGCATTGT GAATCGAGAG AGTGATTCTG CGTAGACTTT TCTGAATGCA
    2551  TTGCGATTCT CTTTTGAATG GATTCTTTGG TTAGGTTTTT CCAGCAGATA
    2601  GCGCTGTTTG TTCTACAATT AAATACCTTG CACACACATT TAAACATGGC
    2651  ATATTTATTA GTCAATTTTG GGAAAAAAAT TAAGCAAATA ACAAGAATCA
    2701  CAAAACTAAT ATTGTGAGTT AATTTGACCA CTTTACAATA AGCCTAGAAT
    2751  GTCATCTACT GTTTAAAACG AGCCTAGAGT GCCCTCTGCT GTTAAACACA
    2801  AGCCTAGAGT GCCCTCTGCT GTTTTAAATA CTATCCTGGA GCTGCATGTA
    2851  CCATTTTAAA TCTAGCCTAG AGCACCGTCG GCTGTTTTAA AACTTGTCTA
    2901  GAGCGACATC TACTGGTGAA AACTTGCCTA GAGCACCTCT GCTGTTTTAA
    2951  ATCTAGCCTA GAGTGCCCTC TGCTGGTGAA ATTTTGCCTA GAGCACCCTC
    3001  TGCTGTTTTA AAACTTGCTT AGAGCGTCAT CTACTGTTGA AAACTTGCCT
    3051  AGAGCACCCT CTGCTGTTTT AAAACTTGCC TAGAGCCCCA TCTGCTAGTG
    3101  AAAACTTGCC TAGAGTGCCC TCTGCTGTTT TAAAACCTAG GCTAGCGTGC
    3151  CTTCTGCTAT TATAAAAATA GCATAGAGCA CCCTGTGCTC TTTTAAAATT
    3201  AGCCTAGAGC ACTATCCGCT GTTTTAAAAC TAGCCTAGAG TGCCCTCTGC
    3251  TGGTGAAAAC TTCCCTAGAG CACCATCTGC TGGGGAAAAC TTCCCTAGAG
    3301  CACCATCTGC TGGTTTAAAA CTTGCCTAGA GTCCCATCTG CTGGTGAAAC
    3351  CTTGCCAAAA GCGCCCTTTG CTGTTTTAAA ACTAGTCTAG AGCTCCATCT
    3401  GCTGGTAAAA ACTAGCCTAA AGCGCCCTCT GCTGATTTAA AACTGGCCTA
    3451  GAGCACCATC TGCTGGGGAA AACTAGCCTA GAGCACCAGC CGCTGTTTTA
    3501  AAACTAGCCT AGATCGCCAT CTGCTGGTGA AAACTAGCCC AGAGCCCCAT
    3551  CTACTGTTAA AAACTAGCCC AGAGCGCACT CTGCTGTTAA AAACTAATCT
                            ActRIIB8
    3601  CAGAATTAAT TTCAGAGTGA ATTGCGATGC ATTTAGAAAA TCTCAGAAAT
    3651  GATCCAGGAA TCTCAATGCT TCAAATTATC GCCCCAGCCT AGTTATCTAC
    3701  ACAGAACATG CAAGACTGTG TGCGTGTGTG CGTGTGCTGA GAAGTTGTTT
    3751  AAATAGAAAA AAAAGGCTAT AAAAAGGGTG TGCGACTGAA AGTAAGTGTT
    3801  TATTTTGGTC GTCTAGTTTC TCTCGTAATG ATGCAATGTG TTTGTTTTGT
    3851  CGTCTGGAGT GAAGCATTCG CACGTCTGAA GCGAATCAGT CCTCATCCAG
    3901  ACTGATAAGG GGGGGGGGGT TCTGGGTCAC GTTTTACATC AAAATTAAAA
    3951  AAAAAAAAAA AAAA
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Fig. 2. (*Continued*)

other clone, LB-1, was obtained from a pre-midblastula transition (< 250-cell) zebrafish embryo cDNA library (Bally-Cuif et al., 1998) through random isolation pro- cedures. This clone contains an insert of 863 bp with sequence identical to nt 301 to 1200 of clone 140-4.

Hydropathy analysis of the deduced amino acid sequence of clone 140-4 revealed two hydrophobic regions: a 19 amino acid stretch at the *N*-terminus corresponding to the signal peptide region and a 27 amino acid transmembrane region. This profile is similar to that of ActRIIB reported for other species, such as mouse (Attisano et al., 1992); *Xenopus* (Mathews et al., 1994), and goldfish (Ge et al., 1997b). The extracellular region has 10 cysteine residues whose positions are identical to other known ActRIIBs (Fig. 3). Similar to other ActRIIBs, the extracellular domain of zebrafish ActRIIB also has two potential glycosylation sites at amino acid positions 42 and 66 (Fig. 3). This region shares 74–91% identity with ActRIIB cloned from human (Hildén et al., 1994), mouse (Attisano et al., 1992), rat (Feng et al., 1993), bovine (Ethier et al., 1997), chicken (Stern et al., 1995), frog (Mathews et al., 1994) and goldfish (Ge et al., 1997b). The transmembrane domain and kinase domain show 67–78% and 82–88% identities, respectively, to other ActRIIBs cloned from human to goldfish. The two regions within the kinase domain, predicted to confer the serine/threonine kinase activity (Mathews and Vale, 1991), are found to be conserved in all vertebrates except the goldfish, in which one amino acid subsititution is found (Fig. 3). Based on these structural features and similarities to other ActRIIBs, we designated this clone (140-4) as the zebrafish activin type IIB receptor (zActRIIB).

A comparison of the 3′ ends of clones E254 and 140-4 showed several deletions ranged from 28 to 307 bp in length in clone 140-4 (Fig. 2). Examination of the sequence in this area revealed that the deletions in clone 140-4 occur between short repeated or highly homologous regions (Fig. 2). This suggests that the deletions found in clone 140-4 likely result from homologous recombination in host cells. To confirm the sequence of ActRIIB cDNA, ovarian cDNA was used as the template in PCR using high fidelity DNA polymerase. Two sets of primers (ActRIIB10 + ActRIIB2 and ActRIIB4 + E254R4, see Fig. 2) which cover the entire coding region, and one set of primer (E254F2 + ActRIIB8) flanking the 3′ end region where deletions in clone 140-4 were found, were used in the PCR. The generated DNA fragments were subjected to direct sequencing. Analysis of the sequencing data confirms that coding sequence in clone 140-4 is correct and that the ovarian cDNA sequence at the 3′ end is identical to that of clone E254. Therefore, nucleotides 1-2700 from clone 140-4 and 1086 to 2349 from clone E254 are assembled and deposited to the Genbank database as the zActRIIB cDNA sequence (accession number AF 069500).

3.2. Tissue distribution of ActRIIB mRNA in adult zebrafish

To determine the tissue distribution of ActRIIB mRNA in adult zebrafish, RT-PCR and subsequent Southern blot analysis were conducted. Using primers located in the coding region, PCR detected a DNA fragment of the expected size in the brain, ovary, testis, muscle, liver and heart (Fig. 4). The ovary showed the highest level of expression while no expression was found in the kidney. In addition, no PCR product was found in the corresponding RNA samples (data not shown), indicating the absence of genomic DNA contamination. To confirm that the PCR cycle used is in the linear range of amplification, ovarian cDNA was subjected to 25, 30, 35, and 40 cycles of PCR and the density of the generated DNA fragments was quantitated. As indicated in Fig. 4C, there is a linear relationship between the PCR cycle number and the density of the PCR product.

Cloning of the mouse (Attisano et al., 1992) and bovine (Ethier et al., 1997) ActRIIB revealed that there are multiple isoforms generated by alternative splicing. In the mouse, alternative splicing of two segments located in the juxtamembrane regions of ActRIIB resulted in the generation of four isoforms, namely ActRIIB1 to IIB4 (Attisano et al., 1992). In the bovine, however, a different segment between the transmembrane domain and the intracellular domain was found to be alternatively spliced, generating another isoform, ActRIIB5 (Ethier et al., 1997). Sequence comparison of zebrafish ActRIIB with mouse ActRIIB indicates that the zebrafish ActRIIB corresponds to the ActRIIB2 isoform. Furthermore, primers used in the tissue distribution study span the alternative splicing regions found in the mouse and bovine and only a single DNA fragment was observed (Fig. 4). These results suggest that zebrafish only express one form of ActRIIB. The RT-PCR experiment was repeated several times with RNA samples prepared from different zebrafish. In all the experiments, we consistently observed a single band.

Since the ovary shows strongest signals in RT-PCR experiments, we further used in situ hybridization to determine the spatial distribution of ActRIIB transcripts in this tissue. Ovarian sections were hybridized

Fig. 3. Deduced amino acid sequence of ActRIIB from zebrafish (z), goldfish (g), *Xenopus* (x), chicken (c), mouse (m), rat (r), human (h) and bovine (b). The predicted hydrophobic signal peptide and transmembrane regions are single and double overlined, respectively. The kinase domain is indicated by arrow heads. Two regions that can be used to predict the serine kinase activity are underlined. Dashes represent identical amino acid residues and dots represent gaps. Ten conserved cysteine residues are indicated by the '△' symbol. The symbol '*' denotes potential glycosylation sites.

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                                                 Δ                    ***     Δ
zActRIIB  MFASLL.TLA LLLATFAADP SHGEVETREC LYYNVNWEVE KTNRSGVER. CEGEKDKRSH
gActRIIB  -F--WP.-F- ---G--S-G- --A----H-- --F-I----- ---------. ----------
xActRIIB  -G--VAL-FL ------R-GS G-D------- I---A---L- ---Q-----L V--K----L-
cActRIIB  -S--W-.--- V-C--LG-G- G---A----- I---A---L- ---Q-----. --------L-
mActRIIB2 -T-PWA.A-- --WGSLC-GS GR--A----- I---A---L- R--Q--L--. ----Q---L-
rActRIIB  -T-PWA.A-- --WGSLC-GS GR--A----- I---A---L- R--Q--L--. ----Q---L-
hActRIIB  -T-PWV.A-- --WGSLWPGS GR--A----- I---A---L- R--Q--L--. ----Q---L-
bActRIIB  -T-PWA.A-- --WGSLC-GS GR--A----- I---A---L- R--Q--L--. ----R---L-

           Δ    ***         Δ          Δ         Δ          ΔΔΔ       Δ
zActRIIB  CYASWRNNSG SIQLVKKGCW LDDFNCYDRQ ECVATEENPQ VFFCCCEGNF CNERFTHLPD
gActRIIB  -------S-- ---------- ---------- ---------- --------D- ----------
xActRIIB  ---------- F-E------- ---------- --I-K----- ---------Y --KK-----E
cActRIIB  ---------- --E------- ---------- ---------- ---------Y ---K-----E
mActRIIB2 -------S-- T-E------- ---------- ---------- -Y-------- ---------E
rActRIIB  -----P-S-- T-E------- ---------- ---------- -Y-------- ---------E
hActRIIB  -----A-S-- T-E------- ---------- ---------- -Y-------- ---------E
bActRIIB  -------S-- T-E------- ---------- ---------- -Y-------- ---------E

zActRIIB  ISGPVI...S PPPVSPSLLN VLVYSLLPLS MLSMAVLLAF WMYRHRKPPY GHVDVNEDPG
gActRIIB  -T---L...E S--SA-L--I --------VT ----LL--G- ---------- ----LS---S
xActRIIB  VE....TFDP K-QPSA-V-N I-I-----IV G----I---- --------S- ---EIN----
cActRIIB  VT--EVIYEP .--PT----N I-------IA V--V-I---- ---------- ----IN----
mActRIIB2 PG--EVTYEP .--TA-T--T V-A-----IG G--LI----- ---------- ----IH----
rActRIIB  PG--EVTYEP .--TA-T--T V-A-----IG G--LI----- ---------- ----IH----
hActRIIB  AG--EVTYEP .--TA-T--T V-A-----IG G--LI----- ---------- ----IH----
bActRIIB  AG--EVTYEP .--TA-T--T V-A-----VG G--LIA---- ---------- --A-IH----

zActRIIB  PSPPSPLVGL KPLQLLEVKA RGRFGCVWKA QMINEYVAVK IFPIQDKLSW QNEREMFSTP
gActRIIB  -------LT- ---------- -----Q--R- --M--C---- -------Q-- ----D-----
xActRIIB  LP-----V-- ------DI-- ---------- RLL------- ---V---Q-- -C-K-I-T--
cActRIIB  -P-------- -------I-- ---------- -LM-D----- -------Q-- -S---I-N--
mActRIIB2 -P-------- -------I-- ---------- -LM-DF---- ---L---Q-- -S---I----
rActRIIB  -P-------- -------I-- ---------- -LM-DF---- ---L---Q-- -S---I----
hActRIIB  -P-------- -------I-- ---------- -LM-DF---- ---L---Q-- -S---I----
bActRIIB  -P-------- -------I-- ---------- -LM-DF---- ---L---Q-- -S---I----

zActRIIB  GMKHDNLLRF IAAEKRG.SN LEMEFWLITE FHERGSLTDY LKGNAVSWAD LCVIAESMAC
gActRIIB  ----E----Y -G--R--.A- --T------- ---------H ----VL--T- --H---T---
xActRIIB  ----E---E- -------.-- ---------A --DK------ -R--L---NE --H-T-T--R
cActRIIB  ----E---Q- -------.T- --T-L----A --DK------ -R--II--NE --HV--T--R
mActRIIB2 ----E---Q- -------.S- --V-L----A --DK------ -R--IIT-NE --HV--T-SR
rActRIIB  ----E---Q- -------CS- --V-L----A --DK------ -R--IIT-NE --HV--T-SR
hActRIIB  ----E---Q- -------.S- --V-L----A --DK------ -R--IIT-NE --HV--T-SR
bActRIIB  ----E---Q- -------.SS --A-L----A --DK------ -R--IIT-NE --HV--T-SR

zActRIIB  GLAYLHEDVP RSKGEGPKPA IAHRDFKSKN VMLKMDLTAV IGDFGLAVRF EPGKPPGDTH
gActRIIB  ---------- --.....--- ---------R- -L--S--S-- L--L------ ---T------
xActRIIB  ---------- -C----H--- ---------- -L-RN----I -A-------- ----------
cActRIIB  --S------- WC----H--- ---------- -L--N----- -A-------- ----------
mActRIIB2 --S------- WCR---H--S ---------- -L--S----- -A-------- ----------
rActRIIB  --S------- WCR---H--S ---------- -L--S----- -A-------- ----------
hActRIIB  --S------- WCR---H--S ---------- -L--S----- -A-------- ----------
bActRIIB  --S------- WCR---H--S ---------- -L--S----- -A-------- ----------

zActRIIB  GQVGTRRYMA PEVLEGAINF QRDSFLRIDM YAMGLVLWEL VSRCKAADGP VDEYMLPFEE
gActRIIB  ---------- ---------- ---------- ---------- ----R----- ----------
xActRIIB  ---------- ---------- ---------- ---------I ----T----- ----L-----
cActRIIB  ---------- ---------- ---A------ ---------- ----R-V--- ----------
mActRIIB2 ---------- ---------- ---A------ ---------- ---------- ----------
rActRIIB  ---------- ---------- ---A------ ---------- ---------- ----------
hActRIIB  ---------- ---------- ---A------ ---------- ---------- ----------
bActRIIB  ---------- ---------- ---A------ ---------- ---------- ----------

zActRIIB  EIGQHPSLED LQDAVVHKKL RPAFKDCWLK HSGLCQMCET MEECWDHDAE ARLSAGCVQE
gActRIIB  ---------- ---------M ---------- ----A----- I--------- --------E-
xActRIIB  ---------- --EV------ --V---H--- -P--A-L-V- I--------- --------E-
cACTRIIB  ---------- --EV-----M --V---H--- -P--A-L-V- I--------- --------E-
mActRIIB2 ---------E --EV-----M --TI--H--- -P--A-L-V- I--------- --------E-
rACTRIIB  ---------E --EV-----M --TI--H--- -P--A-L-V- I--------- --------E-
hACTRIIB  ---------E --EV-----M --TI--H--- -P--A-L-V- I--------- --------E-
bACTRIIB  ---------E --EV-----M ---I--H--- -P--A-L-V- I--------- --------E-

zActRIIB  RISQIRR.VS SSTSDCLFSM VTSLTNVDLP PKESSI
gActRIIB  -------.LT -I-TSD-L-T ---------S ----R-
xActRIIB  ------KS-N GT-----V-I ---V------ ------
cActRIIB  --A---KS-N GT-----V-I ---V------ ------
mActRIIB2 -V-L---S-N GT-----V-L ---V-----L ------
rActRIIB  -V-L---S-N G------V-L ---S-----L ------
hActRIIB  -V-L---S-N GT-----V-L ---V------ ------
bActRIIB  -V-L---S-N GT-----V-L ---V------ ------
```

Fig. 3.

with a DIG-labeled antisense riboprobe and positive staining was found in cytoplasm of oocytes, but not in the follicular cells or interstitial tissues (Fig. 5). ActRIIB mRNAs is expressed in oocytes at all stages of development (stages I–IV, Fig. 5). The intensity of the hybridization signal is inversely related to the size of oocytes; being strongest in stage I and weakest at stage IV. In stage I oocytes (primary growth stage, follicle diameter up to 0.14 mm), intense staining was found in the entire cytoplasm. Strong hybridization signals were also seen in the cytoplasm of stage II oocytes (cortical alveolus stage, follicle diameter = 0.14–0.34 mm). The intensity of signal decreased as oocytes entered stage III (vitellogenesis, follicle diameter = 0.34–0.69 mm) and stage IV (oocyte maturation, follicle diameter = 0.69–0.73 mm). In all stages, hybridization signals were dispersed among cortical alveolus and/or yolk bodies. No hybridization signals were observed in the sense control (data not shown). The experiment has been repeated seven times with ovaries from seven animals, and in all experiments, the same results were observed.

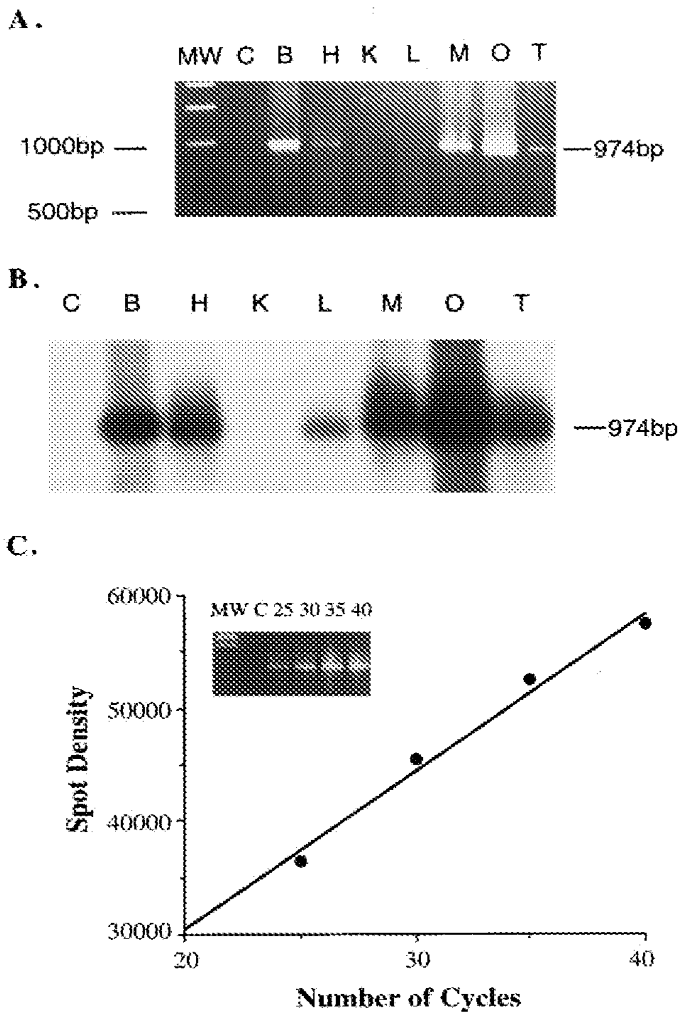

Fig. 4. Distribution of ActRIIB mRNA in various adult zebrafish tissues. Reverse transcription was performed using 2 μg of total RNA isolated from the brain (B), heart (H), kidney (K), liver (L), muscle (M), ovary (O), and testis (T). PCR was performed using primers ActRIIB3 and ActRIIB2. C is the negative control (without the addition of template DNA). (A) An ethidium bromide-stained gel showing the detection of an expected size DNA fragment in several tissues after PCR for 35 cycles. (B) Southern blot hybridization of the PCR products shown in panel A. (C) Validation of PCR. PCR was performed using ovarian cDNA as the template for 25, 30, 35, and 40 cycles and the density of the PCR products was quantified. A linear relationship between cycle number and the density of PCR product can be observed. The insert shows the original ethidium bromide stained gel. C, negative control; number on each lane represents the number of PCR cycles performed.

3.3. Expression of ActRIIB mRNA in embryos

Cloning of ActRIIB from a zebrafish embryonic library suggests expression of ActRIIB mRNA during embryonic development. To determine the spatio-temporal profile of ActRIIB expression during embryogenesis, whole-mount in situ hybridization was carried out under high stringency conditions. Four stages representing major developmental steps were selected for the study: (i) the sphere stage (Fig. 6A, B) which occurs prior to mid-blastula transition, allowing visualization of maternal transcripts; (ii) the shield stage (Fig. 6C, D) which marks the onset of gastrulation; (iii) the tail bud stage (Fig. 6E, F) which is a mid-point in neurulation; and (iv) the 6-7 somite stage (Fig. 6G, H) which corresponds to undergoing somitogenesis. In all these stages examined, intensive signals for ActRIIB mRNA was detected and found to be expressed in a ubiquitous fashion (Fig. 6A, C, E, G). No signal was found in control experiments performed in parallel on the same batches of embryos with the corresponding sense probe (Fig. 6B, D, F, H), demonstrating the specificity of the hybridization signals.

4. Discussion

We have isolated cDNA clones containing the full length sequence of ActRIIB from zebrafish embryonic

Fig. 5. Localization of ActRIIB mRNA in the zebrafish ovary. Ovarian sections were hybridized with DIG-labeled antisense riboprobe for ActRIIB. Specific hybridization signals were detected in cytoplasm of oocytes, from stages I to IV. gv, germinal vesicle. Large arrow head, follicular cells. Small arrow head, interstitial cells. Scale bar: 0.2 mm.

Fig. 6. Whole-mount in situ hybridization of zebrafish embryos with DIG-labeled antisense (A, C, E, G) and sense (negative control, B, D, F, H) probes for ActRIIB. Embryos from four stages of development were used: sphere (A, B), shield (C, D), tail bud (E, F), and 6-7 somite (G, H). ActRIIB mRNA is expressed in all stages examined, in a ubiquitous fashion (A, C, E, G). No signals were found in embryos hybridized with the corresponding sense probe (B, D, F, H). Scale bar: 0.85 mm.

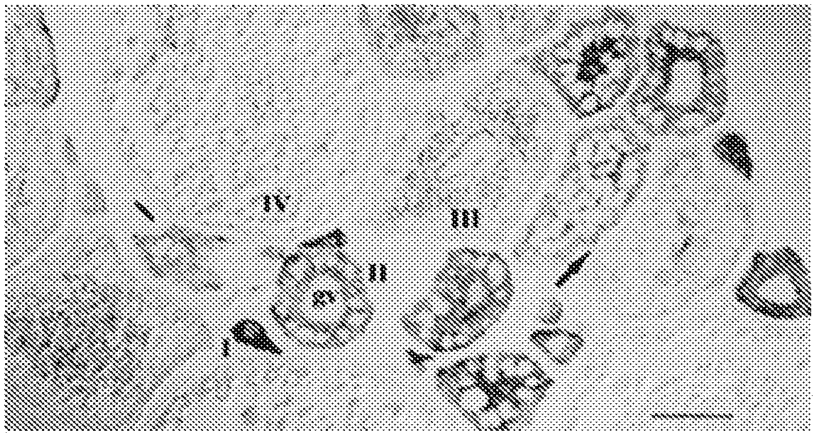

Fig. 5

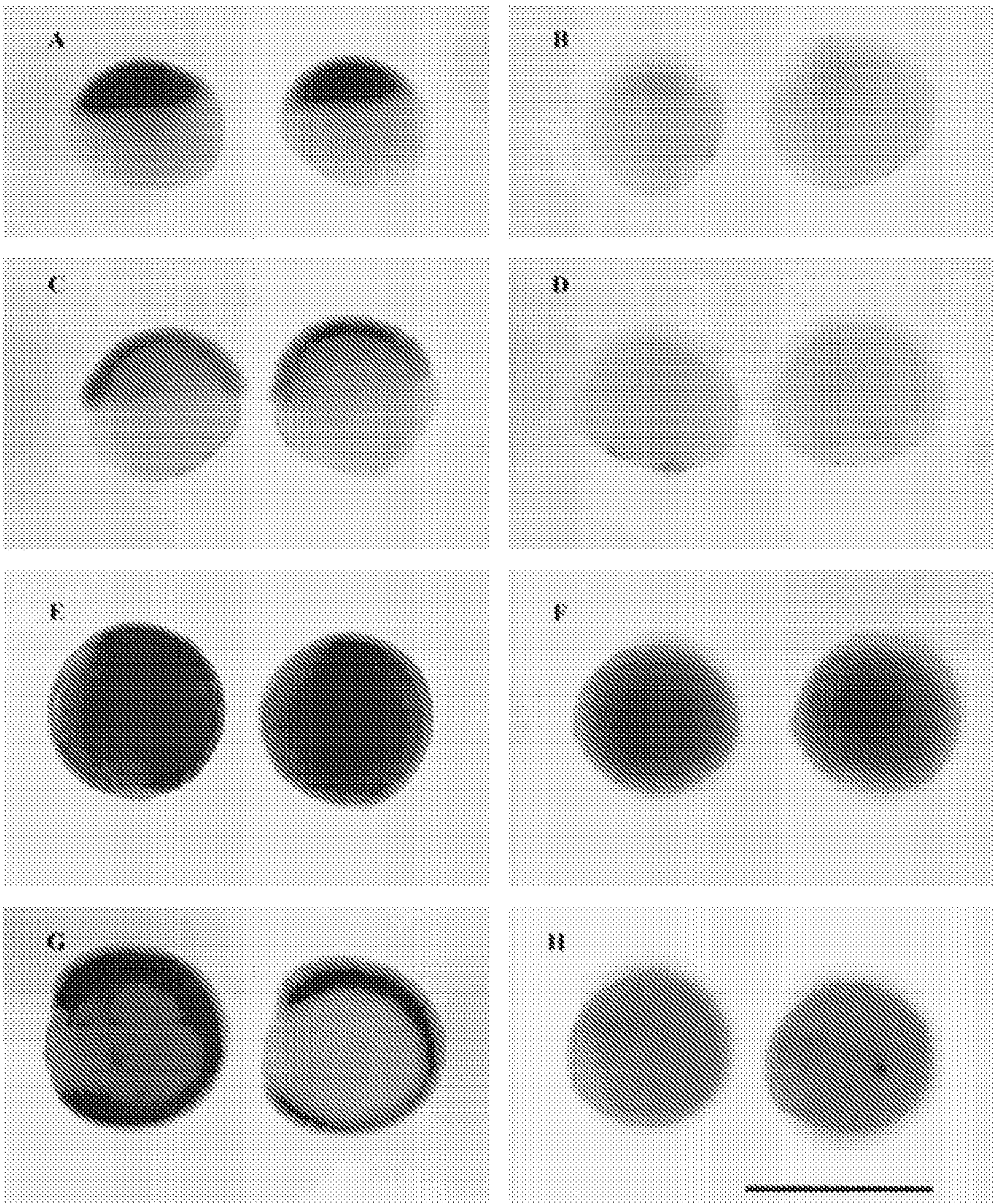

Fig. 6

cDNA libraries. Structural features of this receptor are similar to other members of the TGFβ receptor family (Mathews, 1994; Massagué, 1996). The zebrafish ActRIIB shows a high degree of amino acid identity to ActRIIBs cloned from other vertebrates. Ten cysteine residues and two potential *N*-linked glycosylation sites are found in the extracellular ligand binding domain, which coincides with all other vertebrate species from which ActRIIB has been cloned. This further supports the notion that the cysteine residues and glycosylation may be important in maintaining a three-dimensional structure required for ligand–receptor interaction (Pepin et al., 1996). Several studies have demonstrated that ActRIIB is a functional activin receptor. When cloned ActRIIB is expressed in mammalian cells, it binds to activin with high affinity (Attisano et al., 1992; Ge et al., 1997b). In addition, overexpression of ActRIIB enhances activin-induced gene expression in *Xenopus* embryos (Mathews et al., 1994). On the other hand, over expression of kinase-deficient ActRIIB has been shown to block activin-induced gene expression in several mammalian cell lines (Tsuchida et al., 1995). However, it should also be noted that when co-expressed with a bone morphogenetic protein (BMP) type 1 receptor, ActRIIB can also bind BMP7 (Yamashita et al., 1995).

The zebrafish ActRIIB has a 2.0 kb 3′ UTR while the reported ActRIIB cloned from other species have a 3′-UTR ranging from less than 100–750 bp (Attisano et al., 1992; Feng et al., 1993; Hildén et al., 1994; Mathews et al., 1994; Stern et al., 1995; Ethier et al., 1997; Ge et al., 1997b). It is possible that all the reported ActRIIB clones do not contain a full-length 3′ UTR sequence. Alternatively, the zebrafish ActRIIB clone may represent a longer transcript. Studies in mammals have demonstrated the presence of multiple transcripts for ActRIIB (Feng et al., 1993; Hildén et al., 1994), therefore, it cannot be excluded that zebrafish may have another ActRIIB transcript with a shorter 3′ UTR.

It has been shown that short tandemly repeated sequences may result in deletions and duplications of intervening sequence (Albertini et al., 1982; Lovett et al., 1993). The two isolated zebrafish ActRIIB clones had discrepancies at the 3′ end region. The clone 140-4 has several deletions occurring between direct repeat or highly homologous nucleotides, suggesting that those regions in clone 140-4 have been rearranged during propagation in *E. coli*. Previous studies have shown that deletions can occur at short repeated sequences in both *rec*A$^+$ and *rec*A$^-$ strain background (Albertini et al., 1982; Lovett et al., 1993). The conclusion that clone 140-4 had undergone recombination while clone E254 represents normal 3′-UTR sequence is further supported by the PCR experiment, in which cDNA prepared from adult ovary show identical sequence to clone E254.

The present study demonstrated that only one form of ActRIIB, corresponding to the mouse ActRIIB2 (Attisano et al., 1992), is expressed in the zebrafish. Studies in the mouse (Attisano et al., 1992) and bovine (Ethier et al., 1997) have indicated the presence of multiple isoforms of ActRIIB generated from alternative splicing events. In our studies, however, RT-PCR using primers flanking the reported alternative splicing regions in the mouse and bovine, has consistently resulted in a single PCR product with size corresponding to ActRIIB2. Comparison among ActRIIBs cloned from the zebrafish, goldfish, frog, chicken, rat and human shows that all these ActRIIBs correspond to the ActRIIB2 isoform found in the mouse. In the human, our studies in brain, ovary and placenta have indicated that only the ActRIIB2 is present in these tissues (Peng et al., 1998). Similarly, studies in the human pituitary did not detect any alternative splicing event in the juxtamembrane region (Alexander et al., 1996). It remains to be determined why these alternative splicing events are only specific to mouse or bovine and if the different isoforms mediate different biological activities of activin.

Using RT-PCR, ActRIIB mRNA was found to be expressed in adult zebrafish tissues, including the brain, ovary, testis, muscle, liver and heart. Such wide expression pattern of ActRIIB suggests that activin may have diverse biological functions in fish. Of particular interest is the demonstration that the ovary expresses the highest level of ActRIIB mRNA. Using in situ hybridization, we have further demonstrated that ActRIIB mRNA is present in oocytes at all stages of development. The intensity of hybridization signals in the oocytes decrease as the oocytes increase in size. The decrease in hybridization signals suggests that ActRIIB mRNA expression may decrease with the development of oocytes. Alternatively, this could be due to the dilution of a constant amount of the mRNA as oocytes become larger. Surprisingly, our in situ hybridization did not detect ActRIIB mRNA in follicular cells. This suggests that ActRIIB mRNA is either not expressed in follicular cells or expressed at a very low level that could not be detected by in situ hybridization. This result is somewhat inconsistent with earlier studies in mammals, as Cameron et al. (1994) detected weak hybridization signals of ActRIIB mRNA in both oocytes and granulosa cells in rat ovary. The presence of ActRIIB mRNA in human follicular cells has also been demonstrated by Northern blot analysis (Eramaa et al., 1995) and by PCR (Peng et al., 1998).

The demonstration that zebrafish oocytes express ActRIIB mRNA suggests that oocytes are target cells of activin. This hypothesis is supported by our recent finding that activin induces final oocyte maturation in zebrafish (Garg and Peng, unpublished). Since activin immunoreactivities have been detected in goldfish and

zebrafish ovaries (Ge et al., 1993a; Wittbrodt and Rosa, 1994), it is likely that activin functions as an autocrine/paracrine regulator in the fish ovary. In mammals, activin has been shown to be a local regulator of ovarian functions, such as steroidogenesis, follicular cell growth and differentiation, as well as oocyte maturation (Itoh et al., 1990; Mather et al., 1990; Mauduit et al., 1991; Li et al., 1995; Peng et al., 1996). Whether or not activin regulates follicular cell functions in fish remains to be investigated.

The present study demonstrates that zActRIIB mRNA is widely distributed during early embryonic development. Such expression pattern is consistent with that of several serine/threonine kinase receptors, such as *Xenopus* AR1 (Hemmati-Brivanlou et al., 1992) and zebrafish ALK-8 (Yelick et al., 1998). Several studies have shown that ActRIIB is involved in mesoderm and axis formation. Targeted deletion of ActRIIB gene in the mouse has provided evidence that ActRIIB is important in patterning of the anteroposterior and left-right axes (Oh and Li, 1997). Overexpression of kinase-deficient ActRIIB in *Xenopus* embryos blocks activin-induced mesoderm formation (Hemmati-Brivanlou and Melton, 1992). Similarly, overexpression of mouse activin receptors in zebrafish embryos affected embryonic development (de Vries et al., 1996). The present finding that ActRIIB mRNA is expressed in the zebrafish embryos further supports the notion that ActRIIB is involved in early development of vertebrates.

In summary, cloning of zebrafish activin type IIB receptor provides further information on the structural evolution of activin receptors in vertebrates. It also provides a tool to study the role and signal transduction of activin in lower vertebrates. In the future, a kinase-deficient ActRIIB will be generated and used as a dominant negative inhibitor to investigate the role of this receptor in fish production, particularly during oocyte development and maturation.

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